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## Attenuated PLD1 association and signalling at the H452Y polymorphic form of the 5-HT<sub>2A</sub> receptor

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## Abstract

The 5-HT<sub>2A</sub> receptor (5-HT<sub>2A</sub>R) is implicated in psychotropic changes within the central nervous system (CNS). A number of polymorphisms have been reported in the 5-HT<sub>2A</sub>R gene; one of these results in a non-synonymous change, H452Y, in the carboxy-terminal tail of the receptor protein. The minor allele (9% occurrence) has been statistically associated with CNS dysfunction such as impaired memory processing and resistance to neuroleptic treatment in schizophrenic patients. We investigated the impact of H452Y mutation of the 5-HT<sub>2A</sub>R expressed in COS7 cells on distinctly coupled intracellular signalling pathways from the receptor, focusing on the heterotrimeric G protein-independent phospholipase D (PLD) pathway, compared to the conventional Gq/11-linked phospholipase C (PLC) pathway. The H452Y mutation selectively attenuated PLD signalling, which as in the wild-type receptor, was mediated by a molecular complex involving PLD1 docked to the receptor's carboxy-terminal tail domain. Co-immunoprecipitation and GST-fusion protein experiments revealed that the H452Y mutation selectively reduced PLD1 binding to the receptor. Experiments with blocking peptides to mimic short sections of the 5-HT<sub>2A</sub>R tail sequence revealed that the peptide spanning residue 452 strongly reduced PLD but not PLC responses of the receptor. Similar observations were made when assessing both PLD responses and PLD-dependent cellular proliferation elicited by activation of 5-HT<sub>2A</sub>Rs natively expressed in MCF-7 cells. Overall these findings indicate that the H452Y polymorphic variant of the 5-HT<sub>2A</sub>R displays selective disruption of its PLD signalling pathway. This may potentially play a role in the CNS dysfunction associated with the H452Y allele of the 5-HT<sub>2A</sub>R.

## Keywords

5-HT<sub>2A</sub> receptor; signalling; phospholipase D; phospholipase C; polymorphism; H452Y

## 1. Introduction

The 5-HT<sub>2A</sub> receptor (5-HT<sub>2A</sub>R) is the likely site of action of a number of hallucinogenic drugs and is thought to be an important target of atypical antipsychotic agents, acting as antagonists [1]. In addition to its conventional signalling through Gq/11 to activate phospholipase C (PLC) [2], the 5-HT<sub>2A</sub>R can activate a variety of other signalling pathways [3-9]. There is increasing evidence that the 5-HT<sub>2A</sub>R can also activate phospholipase D (PLD) through small G protein-dependent pathways that may be independent of heterotrimeric G proteins [10, 11]. The 5-HT<sub>2A</sub>R can activate PLD through a molecular complex in which both ARF1 and PLD1 are bound at distinct sites within the receptor's carboxy-terminal domain [11, 12]. In different cell models, the closely related 5-HT<sub>2C</sub>R can activate PLD through a G<sub>13</sub> / Rho - dependant pathway [13]. In the ARF1 / PLD1 - dependant model system investigated here, the binding site for PLD1 has been localised to the distal 33 amino acids of the receptor's carboxy-terminal tail (residues 439-471) [12].

Interestingly the human 5-HT<sub>2A</sub>R gene presents a number of single nucleotide polymorphisms within the coding region, several of which are non-synonymous, for example the H452Y variant (minor allele frequency 9 %; [14, 15]) where the sequence change falls within the region we have identified as the PLD binding site. The H452Y allele has been statistically associated with resistance to the atypical antipsychotics clozapine and olanzapine in schizophrenic patients [16-19], as well as with deficits in memory, novel information processing and neurodevelopment of temporal structures [20-24], with bipolar disorder [25, 26] and perhaps with attention-deficit hyperactivity disorder (ADHD) [27]. There is some evidence that the conventional PLC / Ca<sup>2+</sup> mobilisation pathway of 5-HT<sub>2A</sub>R intracellular signalling may be modified in the H452Y variant [28-30], although other studies did not observe statistically significant alterations in receptor-mediated Ca<sup>2+</sup> responses [31, 32]. It has been suggested that the H452Y variant 5-HT<sub>2A</sub>R may exist in a preferentially desensitised state with respect to PLC signalling and correspondingly displays reduced levels of 5-HT-stimulated [<sup>35</sup>S]GTP-γS binding to pertussis toxin-sensitive and-insensitive G proteins [29]. A modest attenuation of PLD signalling was also seen in these experiments, but under conditions where the 5-HT<sub>2C</sub>R at least utilises a G<sub>13</sub> / Rho -dependant pathway of activation. In addition the H452Y polymorphism may attenuate 5-HT<sub>2A</sub>R homodimerisation and heterodimerisation with the D<sub>2</sub>R, although the functional significance of this is not yet clear [33].

In the present study (primarily utilising a cell model system where the 5-HT<sub>2A</sub>R activates the PLD pathway through an ARF1 / PLD1 complex assembled around its carboxy-terminal tail), we asked whether mutation of residue 452 from the wild-type histidine (H) to the variant tyrosine (Y) form differentially impacts on PLD compared to PLC signalling by the receptor.

## 2. Material and methods

### 2.1. Biochemical reagents

All chemicals were obtained from Sigma-Aldrich, unless otherwise indicated. The selective 5-HT<sub>2A</sub>R agonist (R)-DOI and antagonist M100709 [34] were obtained from RBI-Sigma and Axon Medchem respectively. The selective PLD1 and PLD2 inhibitors VU 0155069 (CAY 10593) and VU 0359595 and the selective PLD2 inhibitor BML 280, which are reported to show 163 fold, 1700 and 21 fold selectivity for their preferred targets in cellular assays [35-37] were obtained from Cayman Chemicals, Avanti Polar Lipids and Enzo Life Sciences respectively. The N-

myristoylated peptides were synthesised by Insight Biotechnology Ltd. Radiolabelled chemicals were obtained from Perkin-Elmer.

## 2.2. Cell culture and transfection

COS7 cells or MCF-7 cells (kindly provided by Nick Morley, University of Edinburgh) were maintained in culture in Dulbecco's modified minimum essential medium (DMEM; Sigma) containing 10 % normal or foetal calf serum, respectively (Harlan) and 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Cells were seeded into 12-well plates (for PLD and PLC assays) or flasks (for ligand binding, co-immunoprecipitation and glutathione S-transferase (GST)-fusion protein studies) and allowed to reach ~ 60 % confluency (after ~ 24 h) before transfer to medium containing defined serum substitute Ultrosor G (Pall Biosepra). Transfections were carried out using FuGENE 6 (Roche Applied Science). Cells were used 72 h after seeding.

## 2.3. Plasmids and mutagenesis

The Protein C epitope-tagged human 5-HT<sub>2A</sub>R construct was described previously [11]; haemagglutinin (HA) epitope-tagged ARF1-HA was kindly provided by Julie Donaldson, NIH; HA epitope-tagged PLD1 and catalytically inactive mutants HA-[K898R]PLD1 and HA-[K758R]PLD2 were kindly provided by Mike Frohman, Stony Brook University; [Q209L/D277N]Gαq was obtained from the Missouri S & T cDNA Resource Centre and [T19N]RhoA was kindly provided by Gary Bokoch (The Scripps Institute). cDNAs for PrC-[H452Y]5-HT<sub>2A</sub>R and GST-[H452Y]5-HT<sub>2A</sub>Rct(376-471) were generated from their wild-type equivalents using the Becton-Dickinson Transformer method for *in vitro* site-directed mutagenesis. In brief, the parent plasmid was denatured to yield two single stranded parent plasmids and incubated with a mutagenic and a selection primer (with a mutation in a restriction enzyme site). The two primers were simultaneously annealed to the single stranded DNA, and following elongation, ligated with T4 DNA ligase. Selected mutated and control plasmids were transformed into *mutS Escherichia Coli (E.Coli)* strains (defective in mismatch repair) and DNA from the pooled bacterial population was isolated. Mutated plasmids were isolated based on the selection primer's resistance to a restriction enzyme. The mutated DNA was then transformed into bacterial cells. Constructs were confirmed by sequencing of both strands.

## 2.4. PLC and PLD assays

PLC and PLD activation was measured by radiotracer assays for [<sup>3</sup>H]inositol phosphate ([<sup>3</sup>H]InsP) production in the presence of 10 mM LiCl and [<sup>3</sup>H]phosphatidyl butanol ([<sup>3</sup>H]PtdBut) production in the presence of 30 mM butan-1-ol, as described previously [10-12, 38]. Standard PLC and PLD assays were carried out over 60 min and 10 min respectively unless otherwise indicated and normally with a 5-HT or (R)-DOI concentration of 3 µM, although 5-HT concentrations from 0.3 nM - 3 µM were used in some experiments. For experiments assessing desensitisation, time-courses of 5-HT-evoked PLC and PLD responses were measured at 6 time points over 0-60 min or 0-15 min respectively.

## 2.5. GST-fusion protein studies

GST-5-HT<sub>2A</sub>R-ct(376-471), GST-[H452Y]-5-HT<sub>2A</sub>Rct(376-471) constructs or empty vector were transformed into BL21-RIL bacterial cells, which were then grown up in standard 2x yeast extract/ tryptone/NaCl medium with 2 % glucose as described

previously [11, 12]. Fusion protein expression was induced by 0.5 mM isopropyl- $\beta$ -D-thiogalactoside (3 h, 37 °C; Invitrogen). Cells were harvested by centrifugation and lysed with BugBuster reagent (Merck Chemicals Ltd.) and centrifuged (12,000 g, 25 min, 4 °C). The supernatant was then incubated for 30 min at room temperature with Glutathione-Sepharose 4B beads (GE Healthcare; 100  $\mu$ l beads with 1:100 protease inhibitor cocktail set III (PI; Calbiochem)/1 ml supernatant), and the matrix was washed extensively with phosphate-buffered saline (PBS). To prepare ARF1-HA and HA-PLD1-enriched cellular extracts, transfected cells were homogenised or lysed respectively in ARF buffer (PBS with 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1:100 PI) or PLD buffer (20 mM HEPES, 150 mM NaCl buffer containing 20 % glycerol, 1 % CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate), 1 % sodium deoxycholate, 1 mM sodium orthovanadate, 1:100 PI). The washed GST-fusion protein-bead complexes were then incubated overnight (4 °C, with rotation) with ARF1-HA or HA-PLD1 extracts or control buffer and PBS. Beads were then washed twice in ice-cold buffer A (PBS containing 20 % glycerol, 1 % CHAPS, 1 % sodium deoxycholate, 0.5 mM EDTA) with 1:100 PI, followed by three washes with diluted buffer A (1:20 in PBS; 1:100 PI). Retained proteins were solubilised in 50  $\mu$ l Laemmli buffer (2 % sodium dodecyl sulphate, 5 % mercaptoethanol, 20 mM Tris-HCl pH 7.4).

## 2.6. Immunoprecipitation and Western blots

Transfected COS7 cells were deprived of serum substitute for 4 h and washed with Hanks' balanced salt solution (10 ml; Invitrogen), prior to the addition of IP buffer (HEPES 20 mM pH 7.5, NaCl 150 mM, 20 % glycerol, 1 % CHAPS, 0.5 % sodium deoxycholate, 1 mM sodium orthovanadate and 1:100 PI; 1 ml/75 cm<sup>2</sup> flask). After 45 min on ice, extracts were centrifuged at 12,000 g (15 min, 4 °C) and the supernatants were pre-cleared with Protein G-Sepharose 4B beads (Sigma; 20  $\mu$ l of 1:1 suspension in IP buffer; incubation: 45 min, 4 °C, with rotation). Samples were then incubated with primary antibodies, as described previously [11, 12]: (mouse anti-Protein C tag; PrC; HPC4, Roche Applied Bioscience or mouse non-immune IgG; NI-IgG; Sigma) and 20  $\mu$ l/ml Protein G-Sepharose 4B suspension before incubation overnight at 4 °C, with rotation. Beads were collected by centrifugation and washed three times with IP buffer prior to the addition of Laemmli buffer (40  $\mu$ l/ml of original supernatant).

Using the NuPAGE Surelock Mini-cell system (Invitrogen), samples and aliquots of original lysate supernatants from immunoprecipitation and GST studies were run on 4 - 12 % Bis-Tris gels, which were subsequently blotted onto polyvinylidene fluoride membrane (Immobilon-P<sup>SQ</sup>; Millipore). Detection antibodies in GST studies were: goat anti-GST (GE Healthcare) used with horseradish peroxidase (HRP)-conjugated donkey anti-goat/sheep IgG (Millipore) and HRP-linked rat anti-HA (3F10). For co-immunoprecipitation studies, detection antibodies were the HRP-linked rat monoclonal anti-HA (3F10; Roche Applied Science) and mouse anti-PrC (HPC4) together with HRP-conjugated donkey anti-mouse secondary (Millipore). The binding of HRP-linked antibodies to membranes was detected by enhanced chemiluminescence using LumiGLO (Cell Signaling Technology). Densitometric grey scale measurements from scanned films were made using Image-J (NIH).

## 2.7. Ligand binding and cell surface biotinylation

After removal of the serum substitute for 4 h, cells were harvested in ice cold ketanserin binding buffer (50 mM Tris, 5 mM MgCl<sub>2</sub>, 1 mM EGTA; pH 7.2 with 1:100 PI and disrupted with an Ystral homogeniser [11, 12, 38]. Homogenates were centrifuged (10 min, 2000 g, 4 °C), to pellet nuclear debris. The supernatants were further centrifuged (30 min, 12,000 g, 4 °C) to pellet microsomal membranes, which

were washed before resuspension by trituration. Membranes were incubated with 0.8 nM [ $^3$ H]ketanserin (72.2 Ci/mmol) and a range of concentrations of unlabelled ketanserin (0.1-300 nM) for homologous displacement, or 10  $\mu$ M mianserin to determine non-specific binding (60 min, 37 °C). Binding was quenched with ice-cold buffer and membranes pelleted by centrifugation (20 min, 12,000 g, 4 °C). The supernatant was aspirated and [ $^3$ H]ketanserin bound to the pellet was measured by scintillation counting. Non-linear curve fitting was used to derive  $K_d$  and  $B_{max}$  values. Protein concentrations were determined using the Pierce BCA Protein Assay Kit.

Cell surface biotinylation was carried out using a membrane-impermeant covalent agent (biotinamidocaproic acid 3-sulpho-N-hydroxysuccinimide ester; NHS-sulfo-biotin) and the capture of solubilised biotinylated proteins on (reversibly binding) monomeric avidin agarose beads as described previously [12]. Biotinylated proteins were eluted using 2 mM biotin in solubilisation buffer. Total and biotinylated receptor levels were assessed by incubating aliquots of lysate and avidin agarose eluate respectively with 0.8 nM [ $^3$ H]ketanserin alone or with 10  $\mu$ M mianserin to determine non-specific binding. Binding was terminated by protein precipitation through the addition of 18 % polyethylene glycol-8000 in PBS at 4 °C with 0.003 % bovine  $\gamma$ -globulin as a carrier. After vigorous vortexing, tubes were kept on ice for 15 min before centrifugation (20 min, 12,000 g, 4 °C). [ $^3$ H]ketanserin associated with the pellets was measured by scintillation counting.

## *2.8 Measurement of cell proliferation using Alamar Blue*

MCF-7 cells were aliquoted into 48-well plates in DMEM containing 0.5% foetal calf serum and penicillin/streptomycin at a density of 30,000 cells/cm<sup>2</sup> and incubated for 24 h to fully adhere. (R)-DOI (3  $\mu$ M) was added to activate 5-HT<sub>2A</sub>R signalling and control wells received only corresponding vehicle. 5-HT<sub>2A</sub>R antagonist and small molecule or peptide blockers of signalling were added as appropriate immediately prior to agonist. Incubation was continued for 48 h at 37°C in 5% CO<sub>2</sub> atmosphere before Alamar Blue solution (Invitrogen) was added to one tenth of the assay volume according to the manufacturer's instructions. After 2 h, fluorescence was measured at excitation 560 nm, emission 590 nm in a fluorometric plate reader. Alamar Blue contains non-fluorescent resazurin, which is converted to bright red-fluorescent resorufin via the reduction reactions of metabolically active cells. The amount of fluorescence produced is proportional to living cell number, so the assay can be used to monitor cell proliferation induced by added stimuli (in this case, the selective 5-HT<sub>2A</sub>R agonist, (R)-DOI).

## *2.9. Data analysis*

Data are presented as mean values  $\pm$  the standard error of the mean (SEM) from *n* separate experiments, as indicated. Where appropriate, data were analysed by non-linear regression (GraphPad Prism 4). The statistical significance of differences was determined by ANOVA, t-tests or Mann-Whitney U-tests as appropriate, with *p* < 0.05 used as the criterion for significance.

## **3. Results**

### *3.1. Comparison of PLC and PLD signalling by the 5-HT<sub>2A</sub>R and the [H452Y]5-HT<sub>2A</sub>R*

As PLD signalling by the 5-HT<sub>2A</sub>R involves PLD1 physically associated with the distal part of the receptor's carboxy-terminal tail, within the span of residues 439-471 [12], we asked whether the H452Y polymorphic form of the receptor might display prominent alterations in PLD signalling compared to its conventional PLC pathway,

coupled through Gq interaction with the third intracellular loop [39]. COS7 cells transiently transfected with the PrC-5-HT<sub>2A</sub>R or the PrC-[H452Y]5-HT<sub>2A</sub>R, expressed similar numbers of specific [<sup>3</sup>H]ketanserin binding sites in the post-nuclear membrane fraction, with similar K<sub>D</sub> values as determined by homologous displacement (Table 1A). In addition, the proportion of specific [<sup>3</sup>H]ketanserin binding sites that could be recovered from monomeric avidin beads after cell surface biotinylation and lysis was similar between PrC-5-HT<sub>2A</sub>R- and PrC-[H452Y]5-HT<sub>2A</sub>R-transfected cells (Table 1B). Thus there appeared to be no marked difference in ligand binding properties or plasma membrane localisation between the two receptor forms. Standard protocol assays for PLC (3  $\mu$ M 5-HT, 60 min) or PLD (3  $\mu$ M 5-HT, 10 min) on COS7 cells transfected with PrC-5-HT<sub>2A</sub>R or PrC-[H452Y]5-HT<sub>2A</sub>R showed significantly reduced PLC and PLD responses in the H452Y variant, with PLD responses reduced to a greater extent; 49% reduction,  $p=0.0044$ , compared to PLC responses; 23% reduction,  $p=0.0422$  (Fig. 1A). Earlier studies on Ca<sup>2+</sup> mobilisation in platelets and PLC responses in transfected NIH3T3 or HEK293 cells indicated attenuation or greater desensitisation of responses in the H452Y variant receptor [28-30]. The relatively modest attenuation of PLC responses seen here in the H452Y variant receptor may relate to the low levels of endogenous arrestin and GRK expression in COS7 cells [40]. When desensitisation of PLC responses was assessed in terms of the time course of [<sup>3</sup>H]InsP production in response to 3  $\mu$ M 5-HT, both the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R showed a gradual reduction in the rate of activity over 0-60 min. Although no statistically significant difference could be detected in the behaviour of the wild type and H452Y variant receptors (Fig.1B), PLC responses of the H452Y variant appeared less than those of the wild-type at longer time points, an observation consistent with Fig. 1A and the idea that desensitisation of PLD signalling may be greater at the H452Y variant. In contrast, the time-course of PLD activation showed a significantly reduced initial rate of [<sup>3</sup>H]PtdBut production at the H452Y variant compared to the wild-type receptor ( $p<0.05$  up to 6 min), (Fig.1D), suggesting that the H452Y polymorphism exerts a mechanistically distinct effect on PLD signalling from that on PLC signalling. 5-HT (3  $\mu$ M) did not elicit any discernible activation of PLC or PLD in untransfected COS7 cells (Fig.1B,C).

### *3.2. PLD signalling at both the 5-HT<sub>2A</sub>R and the [H452Y] variant predominantly involves the PLD1 isoform*

Figure 2 shows that the profile of reduction in 5-HT-induced [<sup>3</sup>H]PtdBut production by the wild-type 5-HT<sub>2A</sub>R due to co-transfection of negative mutant PLD1 but not PLD2 or due to a selective PLD1 inhibitor but not a selective PLD2 inhibitor was fully reproduced at the H452Y variant. This indicated that the H452Y variant receptor utilises a similar pathway of PLD activation as the wild-type 5-HT<sub>2A</sub>R. As reported previously for the wild-type PrC-5-HT<sub>2A</sub>R [12], [<sup>3</sup>H]PtdBut responses of the H452Y variant were not significantly affected by a dominant negative Gq construct, [Q209L/D277N]G $\alpha$ q, pertussis toxin (200 ng/ml overnight, or a dominant negative RhoA construct, [T19N]RhoA, showing  $84 \pm 7$ ,  $92 \pm 10$  and  $83 \pm 9\%$  of control responses respectively (means  $\pm$  SEM,  $n=4$ ). These observations are consistent with a mechanism of PLD activation independent of Gq/11, Gi/o and G13/RhoA, as seen for the wild-type PrC-5-HT<sub>2A</sub>R under similar conditions. We then assessed the effects of co-transfecting additional wild-type PLD1 on [<sup>3</sup>H]PtdBut production responses of the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R. Figure 3 shows that co-transfection of additional wild-type PLD1 increased the concentration-dependent responses to 5-HT of both the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R, although the degree of amplification of response was markedly greater for the PrC-5-HT<sub>2A</sub>R compared to the H452Y variant. Table 2 documents the mean EC<sub>50</sub> and Emax response values from non-linear curve fitting of five independent concentration-response curves in each



case. No significant differences were detected between  $EC_{50}$  values at the PrC-5-HT<sub>2A</sub> R and the PrC-[H452Y]5-HT<sub>2A</sub> R in the absence or presence of additional PLD1.  $E_{max}$  values were significantly greater in the presence of PLD1 for both the wild-type and H452Y variant receptors ( $p < 0.01$  in each case) but the degree of amplification at the PrC-5-HT<sub>2A</sub>R ( $3.10 \pm 0.26$  fold) was significantly greater than at the PrC-[H452Y]5-HT<sub>2A</sub>R ( $1.72 \pm 0.25$  fold;  $p < 0.01$ ).

### *3.3. Physical association of PLD1 with the PrC-[H452Y]5-HT<sub>2A</sub> R through its carboxy-terminal tail is reduced compared to the PrC-5-HT<sub>2A</sub> R*

COS7 cells co-transfected with either the wild-type or H452Y variant receptor, together with HA-tagged PLD1 were solubilised and proteins immunoprecipitated with PrC-tag antibody or non-immune IgG controls. While both the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R were specifically captured at similar levels, the amount of associated HA-PLD1 was clearly and significantly reduced by around 50 % at the H452Y variant compared to the wild-type (Fig.4A, Table 3). This was corroborated in findings with GST-fusion protein constructs of the carboxy-terminal tail domain of the wild-type and H452Y variant receptors (Fig.4B, Table 3). Despite similar input levels of GST-fusion protein constructs, the *in vitro* binding of HA-PLD1 was clearly and significantly reduced by around 50 % at the H452Y variant, whereas binding of ARF1-HA, which occurs at a more proximal segment of the tail domain [11], was unaffected. These observations are consistent with evidence that the critical binding region for PLD1 lies between residues 439-471 [12] and highlight residue 452 as making an important contribution to the binding site, both *in vitro* and in a physiological cellular context.

### *3.4. An N-myristoylated blocking peptide spanning residue 452 selectively disrupts PLD but not PLC signalling by the PrC-5-HT<sub>2A</sub>R*

As the PLD1 binding site on the carboxy-terminal tail of the 5-HT<sub>2A</sub>R falls within the distal 33 residues 439-471, we further tested the role in functional PLD signalling of the region around residue 452 by designing 3 cell-permeable N-myristoylated decoy peptides (replicating residues 439-449, 450-460 and 461-471) and evaluating their effects on cellular PLC and PLD responses of the PrC-5-HT<sub>2A</sub>R in COS7 cells (Fig.5). We have previously shown that similar peptides can enter neuronal cells and effectively disrupt protein:protein interactions of AMPA receptor subunits [41]. Cells were pre-incubated here with the peptides at a concentration of 50  $\mu$ M for 15 min prior to beginning the signalling assays. Neither the 439-449 nor the 461-471 peptide had any discernible effect on either PLC or PLD responses. The 450-460 peptide, encompassing the region around residue 452, had no effect on PrC-5-HT<sub>2A</sub>R PLC responses but strongly attenuated PLD responses ( $p < 0.01$ ). This corroborates our evidence that the region around residue 452 (more specifically residues 450-460) is important in PLD1 association and signalling by the 5-HT<sub>2A</sub> R. The H452Y polymorphism in the 5-HT<sub>2A</sub>R appears to have a physiologically relevant impact selectively on the PLD signalling pathway of the receptor by reducing PLD1 association and activation.

### *3.5. 5-HT<sub>2A</sub>R-mediated PLD signalling and cell proliferation in MCF-7 cells are selectively blocked by PLD1 inhibitors and an N-myristoylated blocking peptide spanning residue 452.*

The 5-HT<sub>2A</sub>R is natively expressed in native tissues and a number of cell lines including MCF-7 breast cancer cells, where it exerts mitogenic effects [42-45]. PLD-mediated generation of phosphatidic acid plays an important role in the activation of mitogenic signalling pathways [46, 47] with 5-HT<sub>2A</sub>R-induced activation of PLD being

specifically implicated in mitogenesis in arterial smooth muscle cells [45]. We therefore investigated 5-HT<sub>2A</sub>R-mediated PLD signalling and proliferation in MCF-7 cells to assess whether residues around amino acid 452 in the carboxy-terminal tail were necessary in a more physiological context. Firstly we carried out ligand binding studies to confirm the presence of 5-HT<sub>2A</sub>R expression in MCF-7 cells, revealing specific [<sup>3</sup>H]ketanserin binding sites with a K<sub>d</sub> of 0.93 ± 0.25 nM and B<sub>max</sub> of 131 ± 28 fmol/mg membrane protein (mean ± SEM, n=3). We then measured PLD signalling responses evoked by the selective 5-HT<sub>2A</sub>R agonist (R)-DOI in MCF-7 cells. Figure 6A shows that (R)-DOI (3 µM)-induced [<sup>3</sup>H]PtdBut production in MCF-7 cells was markedly and significantly inhibited by the selective 5-HT<sub>2A</sub>R antagonist M100907 (200 nM) and the selective PLD1 inhibitors VU 0155069 (0.5 µM) and VU 0359595 (50 nM) but not by the selective PLD2 inhibitor BML 280 (5 µM). This confirms the presence of native 5-HT<sub>2A</sub>R in MCF-7 cells and that they signal through PLD1 rather than PLD2, similar to transfected 5-HT<sub>2A</sub>R in the COS7 cell model. Figure 6B shows that the N-myristoylated decoy peptide mimicking 5-HT<sub>2A</sub>R residues 450-460 (50 µM), but not peptides mimicking residues 439-449 or 461-471 significantly inhibited (R)-DOI (3 µM)-induced [<sup>3</sup>H]PtdBut production in MCF-7 cells. Again this matches results in transfected COS7 cells, indicating in this more physiological context that the region of the 5-HT<sub>2A</sub>R carboxy-terminal tail around residue 452 is crucial for receptor coupling to PLD1. Figure 6C illustrates data on (R)-DOI-induced mitogenesis in MCF-7 cells, as measured by the fluorescent reporter of effective cellular redox function, Alamar Blue (Invitrogen). The (R)-DOI (3 µM)-induced increase in Alamar Blue fluorescence after 48 h growth in DMEM containing 0.5% foetal calf serum [44] was significantly inhibited by the 5-HT<sub>2A</sub>R antagonist M100709 (200 nM), the PLD1 inhibitor VU 0359595 (50 nM) and the N-myristoylated decoy peptide mimicking 5-HT<sub>2A</sub>R residues 450-460 but not those mimicking residues 439-449 or 461-471. These observations indicate that native 5-HT<sub>2A</sub>R in MCF-7 cells appear to signal through PLD1 by a process dependent specifically on residues 450-460 in the receptor carboxy-terminal tail to mediate the physiological response of increased cellular proliferation.

#### 4. Discussion

The present observations support earlier evidence that 5-HT<sub>2A</sub>R-mediated Ca<sup>2+</sup> fluorescence responses were attenuated in platelets of patients heterozygous for the H452Y polymorphism [30] and that PLC signalling appeared reduced or preferentially desensitised in NIH3T3 or HEK293 cells expressing the H452Y mutant receptor [28, 29]. Other reports did not describe such marked effects [31, 32] and the effect on PLC signalling we observed was only modest. This may be due to the cell system used here, transfected COS7 cells, which contain only low levels of native arrestin and GRK [40] and may therefore not display particularly clearly any alternations in desensitisation processes. In contrast we observed a robust inhibition of PLD activation at the H452Y variant of the 5-HT<sub>2A</sub>R with a strongly reduced initial rate of 5-HT-induced [<sup>3</sup>H]PtdBut production. Like the wild-type receptor, the H452Y variant appears to use a mechanism independent of heterotrimeric G proteins to activate PLD1 but not PLD2 and [<sup>3</sup>H]PtdBut production by both receptor variants is selectively amplified in the presence of additional wild-type PLD1. The extent of amplification is however less at the H452Y variant. Correspondingly the direct association of PLD1 with the receptor's carboxy-terminal tail domain seen in co-immunoprecipitation and GST-fusion protein binding experiments is reduced at the H452Y variant receptor compared to the wild-type. The importance of the residues around 452 for PLD docking and signalling was further shown by the selective disruption of 5-HT<sub>2A</sub>R PLD but not PLC signalling by a cell-permeable blocking peptide spanning residues 450-460 but not those spanning other sections of the distal tail domain.

To evaluate whether similar processes occur in cells natively expressing 5-HT<sub>2A</sub>Rs as opposed to the transfected COS7 cell model, we carried out further experiments on MCF-7 human breast cancer cells, which express endogenous 5-HT<sub>2A</sub>Rs and respond to their activation by increased proliferation [44]. We selected this human cell line rather than rodent cell lines or tissues because the distal carboxy-terminal tail of rodent 5-HT<sub>2A</sub>Rs shows significant divergence from the sequence of the human receptor. MCF-7 cells were shown to express moderate levels of [<sup>3</sup>H]ketanserin binding sites and display PLD signalling responses to a selective 5-HT<sub>2A</sub>R agonist that were inhibited by a selective 5-HT<sub>2A</sub>R antagonist, inhibitors of PLD1 but not PLD2 and the myristoylated blocking peptides corresponding to 5-HT<sub>2A</sub>R carboxy-tail residues 450-460 but not residues 439-449 or 461-471. Furthermore the physiological response of 5-HT<sub>2A</sub>R agonist-induced MCF-7 cell proliferation was inhibited by these agents with a closely parallel profile. The importance of the distal carboxy-terminal tail of the 5-HT<sub>2A</sub>R for PLD1 interaction, PLD signalling and downstream cellular responses was therefore confirmed in a more physiological, non-transfected cell context.

Overall these findings provide clear evidence for pathway-selective changes in signalling by a polymorphic form of the 5-HT<sub>2A</sub>R that occurs with moderate frequency in the population and has been associated in molecular genetics studies with a variety of CNS conditions/disorders. While it is possible that such findings may help in validating the potential usefulness of pathway-specific therapeutic interventions, the precise impact the altered signalling is likely to have on CNS neurons is far from clear. 5-HT<sub>2A</sub>Rs are important regulators of pyramidal cell excitability in prefrontal cortex [48]. Altered PLD-mediated production of phosphatidic acid may impact on a number of downstream signalling pathways as well as vesicular trafficking events including exocytosis and endocytosis [49] so effects on neuronal excitability are likely to be complex.

Factors such as disease phenotypes, environment, treatment and cell type-specific regulation of gene expression might potentially also influence allele transcription. It will be difficult to ascribe any of the overall psychiatric/neurological deficits associated with the H452Y polymorphism in the 5-HT<sub>2A</sub>R directly to impairment of its PLD signalling. Nevertheless the evidence that the H452Y allele is associated with poor clinical response in schizophrenic patients to clozapine, which is considered to be the most efficacious antipsychotic agent available [50], emphasises the importance of the 5-HT<sub>2A</sub>R (and perhaps its profile of signalling pathway activation) in psychosis.

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## References

- [1] Dean B, *Journal of Neurochemistry*. 2003;85:1-13.
- [2] Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR, Humphrey PP, *Pharmacological Reviews*. 1994;46:157-203.
- [3] Abbas AI, Yadav PN, Yao WD, Arbuckle MI, Grant SG, Caron MG, Roth BL, *Journal of Neuroscience*. 2009;29:7124-7136.
- [4] Banes A, Florian JA, Watts SW, *The Journal of Pharmacology and Experimental Therapeutics*. 1999;291:1179-1187.
- [5] Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P, Clarke WP, *Molecular Pharmacology*. 1998;54:94-104.

- [6] Guillet-Deniau I, Burnol AF, Girard J, *The Journal of Biological Chemistry*. 1997;272:14825-14829.
- [7] Gonzalez-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q, Sealton SC, Gingrich JA, *Neuron*. 2007;53:439-452.
- [8] Johnson-Farley NN, Kertesz SB, Dubyak GR, Cowen DS, *Journal of Neurochemistry*. 2005;92:72-82.
- [9] Kurrasch-Orbaugh DM, Parrish JC, Watts VJ, Nichols DE, *Journal of Neurochemistry*. 2003;86:980-991.
- [10] Mitchell R, McCulloch D, Lutz E, Johnson M, MacKenzie C, Fennell M, Fink G, Zhou W, Sealton SC, *Nature*. 1998;392:411-414.
- [11] Robertson DN, Johnson MS, Moggach LO, Holland PJ, Lutz EM, Mitchell R, *Molecular Pharmacology*. 2003;64:1239-1250.
- [12] Barclay Z, Dickson L, Robertson DN, Johnson MS, Holland PJ, Rosie R, Sun L, Fleetwood-Walker S, Lutz EM, Mitchell R, *Biochemical Journal*. 2011;436:651-660.
- [13] McGrew L, Chang MS, Sanders-Bush E, *Molecular Pharmacology*. 2002;62:1339-1343.
- [14] Erdmann J, Shimron-Abarbanell D, Rietschel M, Albus M, Maier W, Korner J, Bondy B, Chen K, Shih JC, Knapp M, Propping P, Nothen MM, *Human Genetics*. 1996;97:614-619.
- [15] Serretti A, Drago A, De Ronchi D, *Current Medicinal Chemistry*. 2007;14:2053-2069.
- [16] Arranz MJ, Collier DA, Munro J, Sham P, Kirov G, Sodhi M, Roberts G, Price J, Kerwin RW, *Neuroscience Letters*. 1996;217:177-178.
- [17] Arranz MJ, Munro J, Sham P, Kirov G, Murray RM, Collier DA, Kerwin RW, *Schizophrenia Research*. 1998;32:93-99.
- [18] Masellis M, Basile V, Meltzer HY, Lieberman JA, Sevy S, Macciardi FM, Cola P, Howard A, Badri F, Nothen MM, Kalow W, Kennedy JL, *Neuropsychopharmacology*. 1998;19:123-132.
- [19] Olajosy-Hilkesberger L, Godlewska B, Schosser-Haupt A, Olajosy M, Wojcierowski J, Landowski J, Marmurowska-Michalowska H, Kasper S, *Neuropsychobiology*. 2011;64:202-210.
- [20] de Quervain DJ, Henke K, Aerni A, Coluccia D, Wollmer MA, Hock C, Nitsch RM, Papassotiropoulos A, *Nature Neuroscience*. 2003;6:1141-1142.
- [21] Filippini N, Scassellati C, Boccardi M, Pievani M, Testa C, Bocchio-Chiavetto L, Frisoni GB, Gennarelli M, *European Journal of Human Genetics*. 2006;14:443-449.
- [22] Papassotiropoulos A, Henke K, Aerni A, Coluccia D, Garcia E, Wollmer MA, Huynh KD, Monsch AU, Stahelin HB, Hock C, Nitsch RM, de Quervain DJ, *Neuroreport*. 2005;16:839-842.
- [23] Schott BH, Seidenbecher CI, Richter S, Wustenberg T, Debska-Vielhaber G, Schubert H, Heinze HJ, Richardson-Klavehn A, Duzel E, *PLoS One*. 2011;6:e15984.
- [24] Wagner M, Schuhmacher A, Schwab S, Zobel A, Maier W, *The International Journal of Neuropsychopharmacology*. 2008;11:1163-1167.
- [25] Kumar HB, Purushottam M, Kubendran S, Gayathri P, Mukherjee O, Murthy AR, Ghosh S, Chandra P, Reddy YC, Benegal V, Brahmachari SK, Jain S, *Psychiatric Genetics*. 2007;17:253-260.
- [26] McAuley EZ, Fullerton JM, Blair IP, Donald JA, Mitchell PB, Schofield PR, *Psychiatric Genetics*. 2009;19:244-252.
- [27] Quist JF, Barr CL, Schachar R, Roberts W, Malone M, Tannock R, Basile VS, Beitchman J, Kennedy JL, *Molecular Psychiatry*. 2000;5:537-541.
- [28] Davies MA, Setola V, Strachan RT, Sheffler DJ, Salay E, Hufeisen SJ, Roth BL, *The Pharmacogenomics Journal*. 2006;6:42-51.
- [29] Hazelwood LA, Sanders-Bush E, *Molecular Pharmacology*. 2004;66:1293-1300.
- [30] Ozaki N, Manji H, Lubierman V, Lu SJ, Lappalainen J, Rosenthal NE, Goldman D, *Journal of Neurochemistry*. 1997;68:2186-2193.

- [31] Harvey L, Reid RE, Ma C, Knight PJ, Pfeifer TA, Grigliatti TA, Pharmacogenetics. 2003;13:107-118.
- [32] Reist C, Mazzanti C, Vu R, Fujimoto K, Goldman D, Molecular Psychiatry. 2004;9:871-878.
- [33] Lukasiewicz S, Faron-Gorecka A, Kedracka-Krok S, Dziedzicka-Wasylewska M, European Journal of Pharmacology. 2011;659:114-123.
- [34] Knight AR, Misra A, Quirk K, Benwell K, Revell D, Kennett G, Bickerdike M, Naunyn Schmiedeberg's Archives of Pharmacology. 2004;370:114-123.
- [35] Lavieri R, Scott SA, Lewis JA, Selvy PE, Armstrong MD, Alex Brown H, Lindsley CW, Bioorganic and Medicinal Chemistry Letters. 2009;19:2240-2243.
- [36] Lewis JA, Scott SA, Lavieri R, Buck JR, Selvy PE, Stoops SL, Armstrong MD, Brown HA, Lindsley CW, Bioorganic and Medicinal Chemistry Letters. 2009;19:1916-1920.
- [37] Scott SA, Selvy PE, Buck JR, Cho HP, Criswell TL, Thomas AL, Armstrong MD, Arteaga CL, Lindsley CW, Brown HA, Nature Chemical Biology. 2009;5:108-117.
- [38] Johnson MS, Robertson DN, Holland PJ, Lutz EM, Mitchell R, Cellular Signalling. 2006;18:1793-1800.
- [39] Roth BL, Willins DL, Kristiansen K, Kroeze WK, Pharmacology & Therapeutics. 1998;79:231-257.
- [40] Menard L, Ferguson SS, Zhang J, Lin FT, Lefkowitz RJ, Caron MG, Barak LS, Molecular Pharmacology. 1997;51:800-808.
- [41] Garry EM, Moss A, Rosie R, Delaney A, Mitchell R, Fleetwood-Walker SM, Molecular and Cellular Neuroscience. 2003;24:10-22.
- [42] Nebigil CG, Garnovskaya MN, Spurney RF, Raymond JR, American Journal of Physiology. 1995;268:F122-127.
- [43] Sonier B, Lavigne C, Arseneault M, Ouellette R, Vaillancourt C, Placenta. 2005;26:484-490.
- [44] Sonier B, Arseneault M, Lavigne C, Ouellette RJ, Vaillancourt C, Biochemical and Biophysical Research Communications. 2006;343:1053-1059.
- [45] Liu Y, Fanburg BL, American Journal of Physiology Lung Cellular and Molecular Physiology. 2008;295:L471-478.
- [46] Foster DA, Xu L, Molecular Cancer Research. 2003;1:789-800.
- [47] Zhang Y, Du G, Biochimica et Biophysica Acta. 2009;1791:850-855.
- [48] Puig MV, Gullledge AT, Molecular Neurobiology. 2011;44:449-464.
- [49] Wang X, Devaiah SP, Zhang W, Welti R, Progress in Lipid Research. 2006;45:250-278.
- [50] Davis JM, Chen N, Glick ID, Archives of General Psychiatry. 2003;60:553-564.

**Table 1: Ligand binding to wild-type and H452Y mutant 5-HT<sub>2A</sub>R constructs**

	Specific [ <sup>3</sup> H]ketanserin binding	
	PrC-5-HT <sub>2A</sub> R	PrC-[H452Y]5-HT <sub>2A</sub> R
a) To total membrane fraction:		
K <sub>D</sub> (nM)	0.58 ± 0.05	0.70 ± 0.08
Bmax (pmol/mg protein)	0.90 ± 0.06	0.96 ± 0.08
b) To lysate following cell surface biotinylation:		
Input lysate (dpm/assay)	372 ± 31	336 ± 26
Avidin eluate (dpm/assay)	135 ± 32	154 ± 30
Mean % captured	36	46

A. Affinity and number of binding sites for the selective 5-HT<sub>2A</sub>R antagonist [<sup>3</sup>H]ketanserin at the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R were assessed in post-nuclear membranes of transfected COS7 cells in the absence/presence of 10 μM mianserin, to define non-specific binding. Protein levels were determined using a Pierce BCA Protein Assay Kit. A homologous displacement protocol was used (unlabelled ketanserin concentrations from 0.1 - 300 nM) with non-linear curve fitting to the data from 4 separate determinations. The two 5-HT<sub>2A</sub>R variants were expressed at similar levels and displayed similar affinities for ketanserin. B. Ligand binding to the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R recovered from monomeric avidin beads after cell surface biotinylation and solubilisation in a non-denaturing detergent-containing buffer. [<sup>3</sup>H]ketanserin binding was measured in the absence/presence of 10 μM mianserin in 5 separate determinations. The two 5-HT<sub>2A</sub>R variants showed similar proportions of expression at the plasma membrane.

**Table 2: The effect of additional wild-type PLD1 on the potency and maximal effect of 5-HT-induced [<sup>3</sup>H]PtdBut production responses of the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R**

Receptor variant	5-HT-induced [ <sup>3</sup> H]PtdBut production					
	EC <sub>50</sub> (nM)			E <sub>max</sub> (fold increase over basal)		
	Control	+PLD1	mean ratio	control	+PLD1	mean ratio
PrC-5-HT <sub>2A</sub> R	8.7 ± 1.5	9.3 ± 1.7	1.17 ± 0.44	2.45 ± 0.06	7.89 ± 0.34**	3.10 ± 0.26††
PrC-[H452Y]5-HT <sub>2A</sub> R	9.3 ± 1.5	9.2 ± 2.0	1.20 ± 0.45	1.59 ± 0.14	2.61 ± 0.19**	1.72 ± 0.25

PLD responses to 3  $\mu$ M 5-HT were measured in COS7 cells transfected with the PrC-5-HT<sub>2A</sub>R or PrC-[H452Y]5-HT<sub>2A</sub>R and either wild-type HA-PLD1 or (in controls) empty vector, pcDNA3.1. Data from 5 concentration-response curves in each case were independently fitted by non-linear regression to a sigmoidal dose-response model and mean  $\pm$  SEM values are shown. \*\* p<0.01, significantly greater than the corresponding values in the absence of transfected PLD1 (Mann-Whitney U-test). †† p<0.01, significantly greater than corresponding values for the PrC-[H452Y]5-HT<sub>2A</sub>R (Mann-Whitney U-test).

**Table 3: Direct association of HA-PLD1 with wild-type and H452Y mutant 5-HT<sub>2A</sub>R constructs.**

Assay:	Specific association of HA-PLD1 with bait (Densitometric grey scale units in excess of NI-IgG controls (a) or GST alone (b))	
	5-HT <sub>2A</sub> R	[H452Y]5-HT <sub>2A</sub> R
a) Co-immunoprecipitation with PrC-tagged receptor	71 ± 17	36 ± 20**
b) In vitro binding to GST-fusion proteins of carboxy-tail constructs	56 ± 15	29 ± 11*

The capture of HA-PLD1 immunoreactivity associated with (a) PrC-tagged receptor immunoprecipitates and (b) GST-fusion protein constructs of the receptor carboxy-terminal tail was measured by quantitative densitometry of Western Blot films. In a) values for wild-type and H452Y mutant 5-HT<sub>2A</sub>R receptors in excess of control pull-downs with non-immune IgG were compared (n=4); \*\* indicates p=0.0027, significantly less than wild-type (paired Student's t-test). In b) values for wild-type and H452Y mutant 5-HT<sub>2A</sub>R carboxy-terminal tail GST-fusion protein constructs in excess of GST alone were compared (n=5); \* indicates p=0.0188, significantly less than wild-type (paired Student's t-test).



## Figure legends

**Fig. 1.** Effects of H452Y mutation of the PrC-5-HT<sub>2A</sub>R on 5-HT-induced PLC and PLD responses in transfected COS7 cells. A. In standard protocol assays, 3  $\mu$ M 5-HT-evoked responses were measured (60 min for PLC, 10 min for PLD) in 6 independent experiments. Data are displayed as mean  $\pm$  SEM. Analysis by two-tailed Student's t-test indicated significantly reduced PLC responses (\*,  $p=0.0422$ ) and PLD responses (\*\*,  $p=0.0044$ ) for the H452Y variant compared to the wild-type receptor, despite similar expression levels and affinity (Table 1). B. Time-course of 5-HT-induced PLC responses did not reveal a statistically significant difference between the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R (means  $\pm$  SEM,  $n=4$ , 2-Way ANOVA with Bonferroni *post-hoc* test). C. Time-course of PLD responses showed a statistically significant attenuation of 5-HT-induced [<sup>3</sup>H]PtdBut production from 0-6 min in the PrC-[H452Y]5-HT<sub>2A</sub>R compared to the PrC-5-HT<sub>2A</sub>R (\*,  $p<0.05$ , mean  $\pm$  SEM,  $n=4$ , 2-Way ANOVA with Bonferroni *post-hoc* test).

**Fig. 2.** Effect of dominant negative PLD1 or PLD2 constructs as well as selective PLD1 or PLD2 inhibitors on 5-HT-induced [<sup>3</sup>H]PtdBut production responses at the PrC-5-HT<sub>2A</sub>R or the PrC-[H452Y]5-HT<sub>2A</sub>R in standard assay conditions. Catalytically inactive mutant PLD1 and the selective PLD1 inhibitor VU 0155069 (CAY 10593) at a concentration of 0.5  $\mu$ M caused statistically significant inhibition of responses to 3  $\mu$ M 5-HT (\*\*,  $p<0.01$  ANOVA with Dunnett's *post-hoc* test) similarly at both the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R ( $n=5$  in each case). Catalytically inactive PLD2 or the selective PLD2 inhibitor BML 280 at a concentration of 5  $\mu$ M had no discernible effect.

**Fig.3.** Effect of transfected additional wild-type PLD1 on 5-HT-induced [<sup>3</sup>H]PtdBut responses of the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R. Responses to 5-HT (0.3 nM - 3 $\mu$ M) were measured in duplicate in 5 separate experiments for the PrC-5-HT<sub>2A</sub>R and the PrC-[H452Y]5-HT<sub>2A</sub>R in cells co-transfected with empty vector pcDNA3.1 or wild-type HA-PLD1. Data are plotted as the overall mean  $\pm$  SEM values from each composite data set ( $n=5$ ). Individual data sets were fitted by non-linear regression and the curve parameters are displayed and statistically evaluated in Table 2. Additional wild-type PLD1 amplified [<sup>3</sup>H]PtdBut production responses at both the PrC-5-HT<sub>2A</sub>R and its H452Y variant but the degree of amplification was notably greater at the wild-type receptor.

**Fig.4.** Specific association of PLD1 with the PrC-5-HT<sub>2A</sub>R in co-immunoprecipitation and GST-fusion protein binding experiments is reduced in the H452Y variant. A. COS7 cells co-transfected with either the PrC-5-HT<sub>2A</sub>R or the PrC-[H452Y]5-HT<sub>2A</sub>R and HA-PLD1 were solubilised and PrC-tag-containing proteins were pulled down with a specific mouse monoclonal antibody (HPC4), or non-immune IgG for controls, together with Protein G-Sepharose beads. Captured proteins were separated by SDS-PAGE and blots were probed for PrC and HA tag immunoreactivity. Despite similar input levels of PrC-tagged receptors and HA-PLD1, the amount of HA-PLD1 specifically captured in association with the H452Y variant receptor was clearly reduced compared to the wild-type PrC-5-HT<sub>2A</sub>R. The double-ended arrow indicates that the HA-PLD1 lysate blot images for lanes 1 and 2 apply similarly to lanes 3 and 4 as each lysate was split between PrC-tag pulldown and NI-IgG control procedures. B. GST-fusion protein constructs of the carboxy-terminal tail domain of wild-type or H452Y variant receptors (residues 376-471) or GST alone were attached to glutathione-Sepharose beads and incubated with extracts of COS7 cells expressing HA-PLD1 or ARF1-HA. Despite similar input levels of constructs and similar binding of ARF1-HA, the H452Y variant receptor construct clearly bound lower levels of HA-PLD1 than the wild-type.

**Fig. 5.** Effects of N-myristoylated (cell-permeable) blocking peptides spanning different segments of the receptor's distal carboxy-terminal tail domain on PLC and PLD responses of COS7 cells expressing the PrC-5-HT<sub>2A</sub>R. 5-HT (3  $\mu$ M)-induced [<sup>3</sup>H]InsP and [<sup>3</sup>H]PtdBut production were measured in cells using the standard assay protocols after 15 min pre-incubation with the different peptides at a concentration of 50  $\mu$ M. Data are shown as mean  $\pm$  SEM values from 5 separate experiments in each case. The peptide covering residues 450-460 (which encompasses residue 452) caused a clear and selective reduction in PLD but not PLC responses (\*\*  $p < 0.01$ , by ANOVA with Dunnett's *post-hoc* test). Neither the residue 439-449 peptide nor the residue 461-471 peptide had any discernible effect.

**Fig. 6.** Effects of 5-HT<sub>2A</sub>R antagonist, PLD inhibitors and N-myristoylated blocking peptides on 5-HT<sub>2A</sub>R agonist-induced PLD signalling and proliferation of MCF-7 cells. A. [<sup>3</sup>H]PtdBut production responses induced by 3  $\mu$ M (R)-DOI were significantly inhibited by the selective 5-HT<sub>2A</sub>R antagonist M100709 (200 nM) or the selective PLD1 inhibitors VU 0155069 (0.5  $\mu$ M) and VU 0359595 (50 nM) but not by the selective PLD2 inhibitor BML 280 (5  $\mu$ M); means  $\pm$  SEM,  $n = 4$ , \* $p < 0.05$  or \*\* $p < 0.01$  by ANOVA with Dunnett's *post-hoc* test. B. [<sup>3</sup>H]PtdBut production responses induced by 3  $\mu$ M (R)-DOI were significantly inhibited by the N-myristoylated blocking peptide mimicking 5-HT<sub>2A</sub>R residues 450-460 but not those covering residues 439-449 or 461-471 (50  $\mu$ M with 15 min pre-incubation); means  $\pm$  SEM,  $n = 4$ , \* $p < 0.05$  by ANOVA with Dunnett's *post-hoc* test. C. (R)-DOI (3  $\mu$ M)-induced increases in Alamar Blue fluorescence of MCF-7 cells, reflecting additional cell proliferation over 48 h incubation with the 5-HT<sub>2A</sub>R agonist were significantly inhibited by M100907 (200 nM), VU 0359595 (50 nM) and the N-myristoylated peptide for residues 450-460 but not those for residues 439-449 or 461-471 (50  $\mu$ M). Values are means  $\pm$  SEM,  $n = 4$ , \* indicates  $p < 0.05$  by ANOVA with Dunnett's *post-hoc* test. Under the present conditions the blockers had no significant effect on proliferation in the absence of 5-HT<sub>2A</sub>R agonist.

Fig. 1.

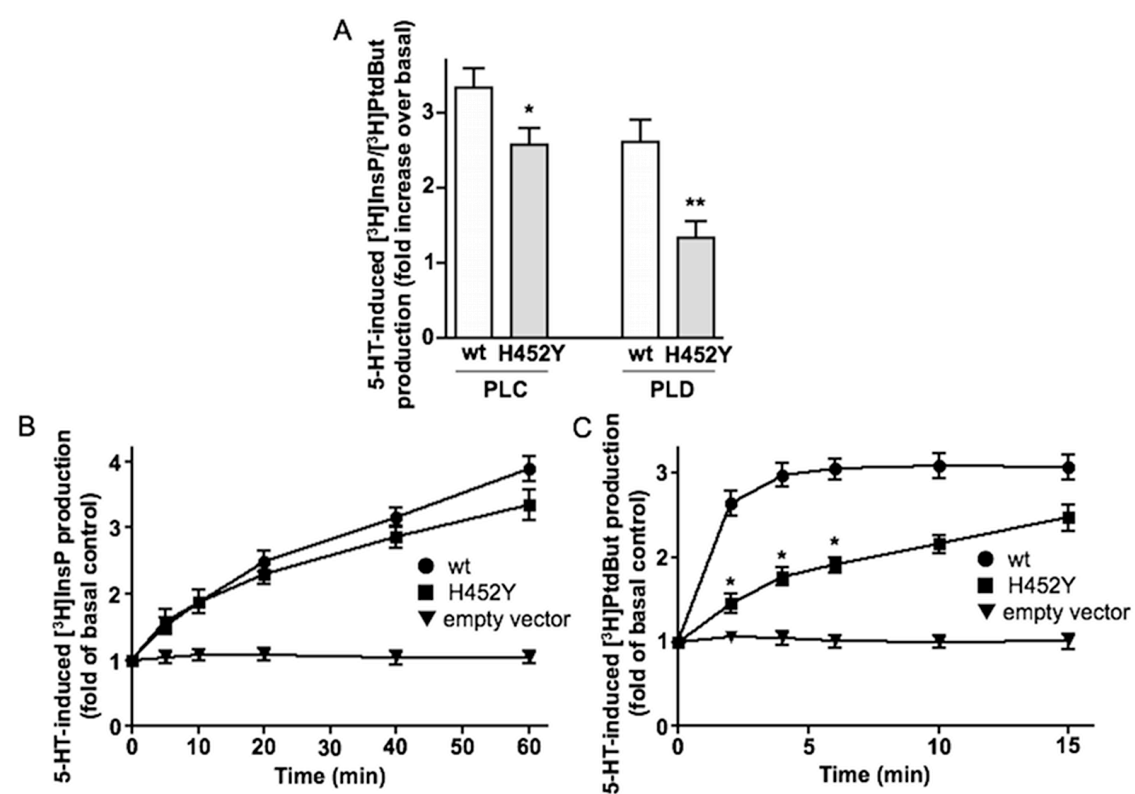


Fig. 2.

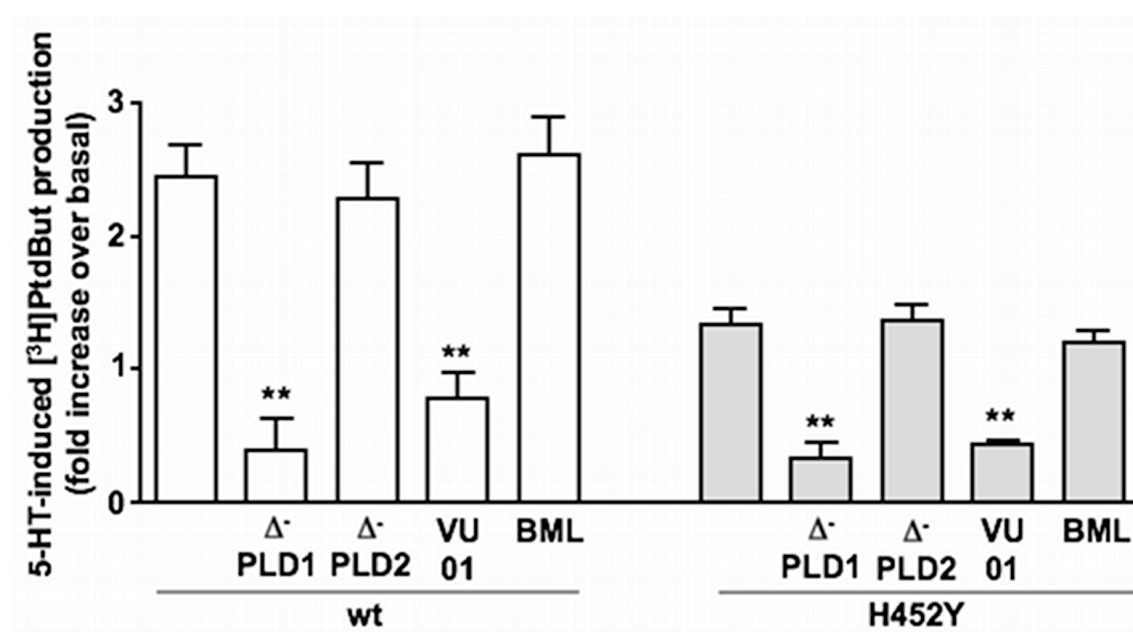


Fig. 3.

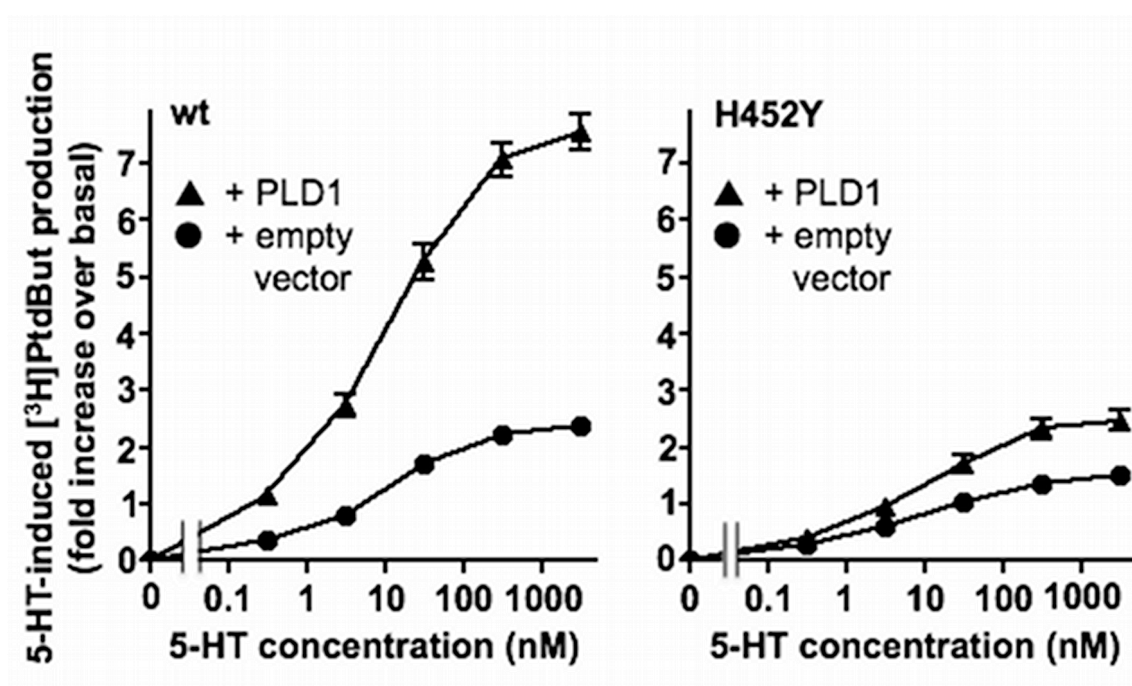


Fig. 4.

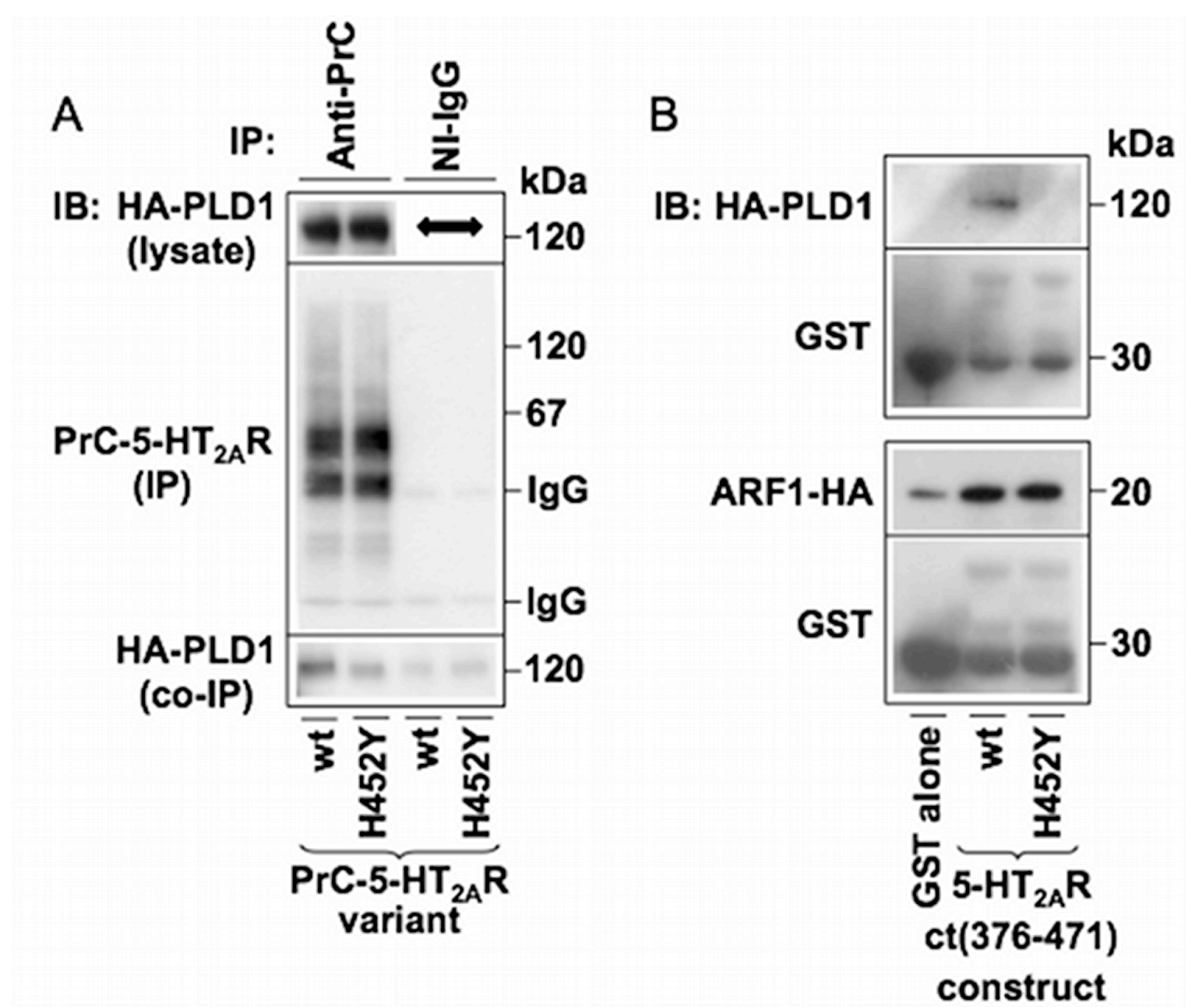


Fig. 5.

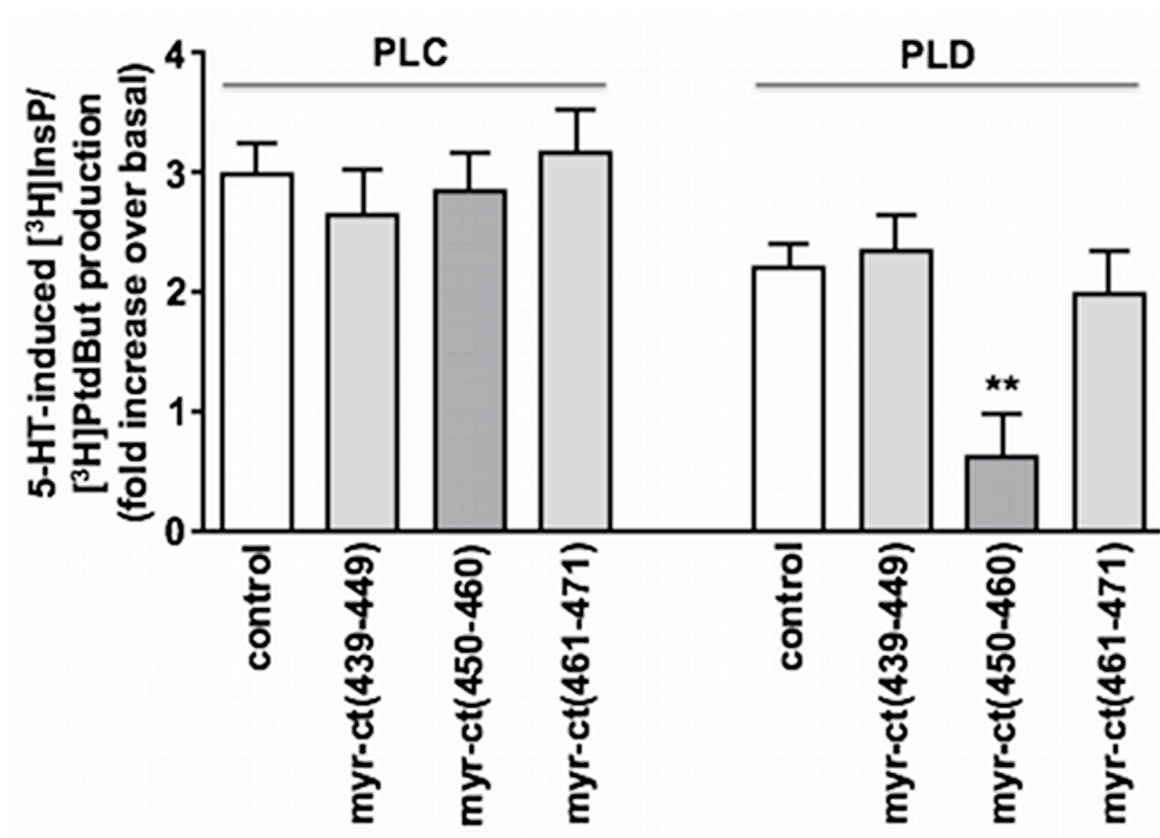


Fig. 6.

